Martin-de-Londres (France), and 13 (all different from the first 15) in a sample of 28 mosquitoes from Sintra (Portugal). None of these were unamplified B1 or B2 alleles.

These observations suggest that all amplified esterase B2 genes and flanking sequences have arisen from a single initial event and support the hypothesis that the worldwide presence of esterase B2 amplification is due to recent migration. This conclusion agrees well with the strong linkage disequilibrium, also observed worldwide, between esterases B2 and A2, which can best be explained by a rapid migration of an initial A2–B2 gene association. The place and time of the occurrence of the original amplification event cannot be traced, but Africa or Asia is most likely[4,11]. The recent history of the sudden appearance of esterases A2–B2 is well documented for California, Italy and France[3,12,13] and a migration between continents is not unlikely, as live larvae and adults of the *C. pipiens* complex are often found on international flights and boats[14–16].

The rapid spread of B2 esterase gene amplification since its probable occurrence in the 1960s (when the intensive use of organophosphate insecticides began) can be compared with that of the *Drosophila melanogaster* alcohol dehydrogenase *Fast* allele associated with high alcohol dehydrogenase activity. There is less restriction polymorphism among *Fast* than *Slow* alleles[17–20]. This has been interpreted to reflect a recent origin of the *Fast* allele, perhaps within the past 2,000 years[17]. Its presence, mainly in temperate areas, may result from an extensive migration and an unknown selective factor.

Resistance genes (or genetic factors such as gene amplification) may arise in a population under insecticide control by mutation or by migration. In *C. pipiens*, very few factors conferring significant organophosphate resistance have been selected during the past 30 years, indicating that the genetic possibilities are limited. As each has a relatively low probability of occurrence, the evolution of organophosphate resistance in a treated population is more likely to be determined by the extent of migration rather than by the rate of mutation. □

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Action potentials must admit calcium to evoke transmitter release

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THERE are two hypotheses to explain how neurons release transmitter. The calcium hypothesis proposes that membrane depolarization is necessary only for opening calcium channels and increasing internal calcium concentration ($[Ca^{2+}]_i$) near membrane transmitter-release sites[1–3]. These calcium ions trigger a transient release of neurotransmitter[4,5]. The calcium-voltage hypothesis postulates that voltage induces a conformational change in a membrane protein rendering it sensitive to calcium such that, in the presence of high $[Ca^{2+}]_i$, depolarization directly triggers transmitter release[6–9]. Here we report that when calcium influx is blocked by cobalt or manganese ions in a calcium-free Ringer, as measured with Fura-2, and $[Ca^{2+}]_i$ is elevated by liberation from a caged calcium compound, transmitter release at the crayfish neuromuscular junction is unaffected by presynaptic action potentials. These results support the calcium hypothesis.

To distinguish between the hypotheses we had to independently increase $[Ca^{2+}]_i$ in terminals and depolarize the membrane without allowing calcium influx. New photolabile 'caged' calcium compounds decrease their affinity for calcium on exposure to ultraviolet light and permit the elevation of $[Ca^{2+}]_i$ in presynaptic terminals without depolarizing the membrane. A direct effect of voltage on transmitter release at crayfish neuromuscular junctions was inferred recently[10] from postsynaptic responses to presynaptic action potentials in low-calcium solutions containing calcium-channel blockers, following elevation of presynaptic $[Ca^{2+}]_i$ with the photosensitive calcium chelator nitr-5 or the mitochondrial poison carbonyl cyanide *m*-chlorophenylhydrazine. Upon testing these low-calcium solutions used in ref. 10 by using the calcium indicator dye Fura-2, we found that calcium influx was not fully blocked, and transmitter release was clearly evoked by presynaptic action potentials at high stimulus frequencies. Therefore, the conclusion that voltage has a direct effect on transmitter release is invalid, because membrane depolarization activated calcium influx. We reexamined the calcium-voltage hypothesis at the crayfish neuromuscular junction using the photolabile calcium chelator DM-nitrophen and tested solutions containing calcium-channel blockers using the calcium indicator Fura-2 to be certain calcium influx was abolished during membrane depolarization.

The fluorescent dye Fura-2 was iontophoresed into the excitatory motor axon of the first walking leg of the crayfish, *Procambarus clarkii*, and intracellular $[Ca^{2+}]_i$ in presynaptic boutons was determined ratiometrically from signals obtained with 350 and 385 nm illumination. Excitatory postsynaptic potentials (e.p.s.ps) were recorded simultaneously in muscle fibres where the imaged terminals synapsed or in adjacent fibres. An extracellular electrode on an axonal branch recorded nerve field potentials to confirm action potential invasion of terminals. A nerve field potential was also sometimes observed in the postsynaptic recording. We used the same stimulation paradigm employed in ref. 10 (twin pulses, 20 ms apart delivered at 10 Hz) as well as high frequency stimulation at 100 Hz.

Figure 1*a* exemplifies experiments in which $[Ca^{2+}]_i$ rose by 87 ± 17 nM (mean ± s.d., 21 terminals in 10 preparations designated $N = 21, 10$) during a 2-min twin-pulse stimulation in 'calcium-free Ringer's solution' containing 12.5 mM magnesium. Even though there was a small calcium influx at this frequency, transmitter release was undetectable, owing to the highly nonlinear relationship between calcium and release[11] (Fig. 1*b* and

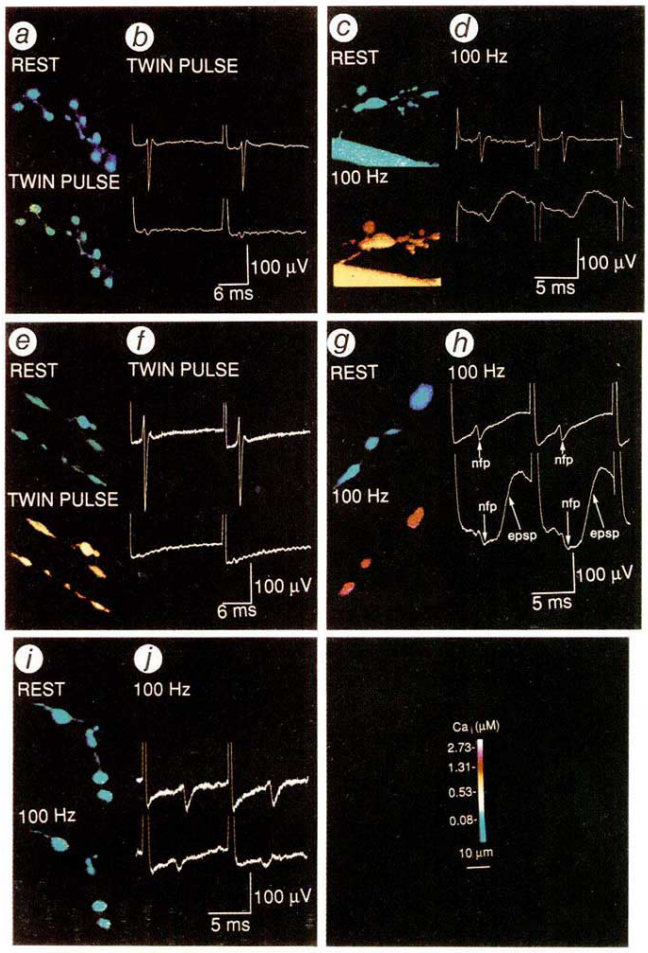

FIG. 1 Measurements of intracellular calcium and evoked transmitter release during presynaptic stimulation in 'calcium-free Ringer's' containing 12.5 mM Mg (*a–d*), 'manganese Ringer's' containing 0.2 mM Ca, 2 mM Mn, and 12.5 mM Mg (*e–h*), or 'cobalt Ringer's' containing 13.5 mM Co and 30 mM Mg (*i, j*). Each panel is from a different preparation. *a, e*, $[Ca^{2+}]_i$ measured at rest and during twin-pulse stimulation and *c, g, i*, 100 Hz stimulation. *b, f*, Averaged e.p.s.ps (bottom trace) and nerve field potentials (n.f.ps, top trace) recorded during twin-pulse stimulation and *d, h, j*, during 100 Hz stimulation. Neither calcium-free magnesium Ringer's nor manganese Ringer's with some calcium added prevented calcium influx and transmitter release; the cobalt Ringer's with no added calcium prevented both.

METHODS. The excitatory axon of the first walking leg of the crayfish (*Procambarus clarkii*), was injected with Fura-2 as described in ref. 13. Individual presynaptic terminals were imaged using a ×40 Zeiss water immersion lens and Nikon Optiphot microscope equipped with a Dage silicon intensified target camera (Model 66). $[Ca^{2+}]_i$ was calculated from 'ratioed' fluorescence images with excitation at 350 and 385 nm (ref. 13). The excitatory axon was stimulated with a suction electrode placed on the nerve dissected from the meropodite. The e.p.s.ps were recorded with an intracellular electrode containing 3 M KCl (10 MΩ) placed in a muscle fibre where imaged terminals synapsed. An extracellular electrode (25–50 μm diameter), placed on an axonal branch on the surface of the opener muscle, recorded the nerve field-potential at its time of arrival in this particular branch. The e.p.s.ps and motor nerve field-potentials were averaged on a digital oscilloscope (Nicolet 4094, Madison). Normal Ringer's contained (in mM) 195 NaCl, 13.5 CaCl, 5.4 KCl, 2.6 MgCl, and 10 sodium–HEPES at pH 7.4. Temperature was maintained at 18 °C. A gravity perfusion system with inflow above the opener muscle provided continuous perfusion with the appropriate saline solution. To ensure complete removal of calcium from the bath, the objective was lifted out of the bath and the muscle surface washed vigorously with a syringe having a 1-mm-diameter plastic tube attached to the tip. A perfusion time of 5–10 min was needed to eliminate calcium influx during a high-frequency train. We found that a manganese Ringer's with no added calcium was equivalent to the cobalt Ringer's in preventing calcium influx. When both calcium and manganese were present in the Ringer, a rise in $[Ca^{2+}]_i$ was measurable for only 20 s at 100 Hz. Manganese ions appear to pass through calcium channels in presynaptic terminals[14] and then bind to Fura-2 and cause quenching of its fluorescence[15–17]. From the intensity of Fura-2 fluorescence, we estimate its concentration at 100–150 μM in boutons. Complete quenching suggests that about 100 μM manganese accumulates during a 20 s, 100 Hz train. This may be compared to a rise in free $[Ca^{2+}]_i$ of over 5 μM at 100 Hz in normal Ringer's[13]. About 99% of entering calcium is bound to cytoplasmic buffers[18], so the influx of manganese through calcium channels is less than 20% that of calcium. We preferred the cobalt Ringer's because quenching of the Fura-2 signal took longer (40–60 s), suggesting that cobalt is less permeable than manganese through calcium channels.

ref. 10). At 100 Hz for 2 min, $[Ca^{2+}]_i$ increased from a resting value of 104 ± 45 nM to approximately 923 ± 313 nM ($N = 9, 5$) (Fig. 1*c*). This large increase in $[Ca^{2+}]_i$ facilitated transmitter release sufficiently that it was now easily detectable (Fig. 1*d*). When such experiments were done using a 'manganese Ringer's' containing 0.2 mM calcium, 2 mM manganese, and 12.5 mM magnesium, a large increase in $[Ca^{2+}]_i$ was seen at frequencies

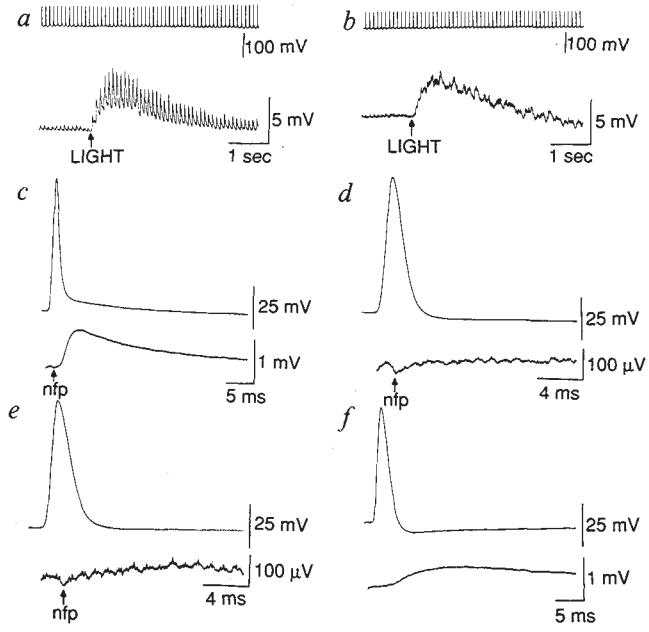

FIG. 2 Effect of action potentials on transmitter release in the absence of calcium entry. In each panel, the upper trace shows presynaptic action potentials stimulated at 10 Hz and the lower trace shows postsynaptic potentials. *a*, control experiment on a preparation injected presynaptically with DM-nitrophen and bathed in normal calcium solution. Photolysis with a 10-s-exposure to UV light beginning at the arrow elevated presynaptic $[Ca^{2+}]_i$ and triggered transmitter release, seen as a depolarizing shift in the postsynaptic baseline. Spike-evoked e.p.s.ps were also strongly facilitated. The gradual decline of the response probably reflects active extrusion of the photo-released calcium from the terminals and depletion of releasable transmitter quanta. The remaining five panels are from a different preparation showing that action potentials evoked no transmitter release in cobalt Ringer which effectively prevents calcium influx. *b*, Presynaptically injected nitrophen was photolysed to trigger transmitter release directly. Both before and during the light exposure, presynaptic spikes evoked no release in cobalt Ringer's. *c–f*, Averages of 50 responses to presynaptic stimulation in the experiment of panel *b*. *c*, Normal e.p.s.ps evoked by action potentials in normal Ringer's. A small field potential reflecting invasion of the presynaptic terminals by action potentials preceded the e.p.s.p. *d*, E.p.s.ps blocked by cobalt Ringer's but nerve terminal potential remaining, indicating that presynaptic terminals were still invaded. *e*, First 50 responses during photolysis of the nitrophen. The nerve terminal potential was still evident, and the increased noise reflected an acceleration in miniature e.p.s.p. frequency following elevation of $[Ca^{2+}]_i$. Nevertheless, action potentials evoked no transmitter release in the absence of calcium influx. When normal extracellular calcium was restored (*f*), action potentials again admitted calcium presynaptically, and e.p.s.ps returned.

METHODS. Bevelled microelectrodes were filled with 60 mM DM-nitrophen, 18 or 36 mM $CaCl_2$, and 10 mM sulphorhodamine B (Molecular Probes) in 0.5 M potassium–HEPES, pH 7.3. The exciter axon was impaled near the Y-branch on the muscle surface and filled by pressure injection as described in ref. 19. By including 10 mM sulphorhodamine B (Molecular Probes) in the electrode, we could monitor filling the terminals without photolysing the DM-nitrophen, which reached a final concentration of about 3 mM. Light from a 100 W mercury epifluorescence illuminator was attenuated with a 25% transmission N.D. filter and focused onto terminals through a ×10, 0.25 n.a. objective to photolyse about 20% of the nitrophen within 1 s, raising $[Ca^{2+}]_i$ from 0.1–0.5 μM to several micromolar. The exact levels of $[Ca^{2+}]_i$ depend on concentrations of magnesium, ATP, DM-nitrophen, and native calcium buffer in nerve terminals[19], and are presently undetermined. When nitrophen was injected without calcium loading in calcium-free media, photolysis evoked little or no transmitter release, indicating that release of magnesium from nitrophen does not trigger the responses. The injection electrode recorded the presynaptic action potential and a postsynaptic electrode was placed in a muscle fibre onto which filled terminals synapsed and from which a motor nerve field-potential could be recorded. Miniature e.p.s.p. frequency was estimated from the postsynaptic depolarization as described in ref. 5. A calcium-activated potassium current ($I_{K(Ca)}$) has been reported in crayfish presynaptic terminals[20]. To prevent shunting of action potentials following $[Ca^{2+}]_i$ elevation by nitrophen photolysis, tetraethylammonium was added to cobalt Ringer's at 2 mM, a dose which is effective in blocking $I_{K(Ca)}$ (M. Wojtowicz, personal communication).

of 100 Hz, from 69 ± 25 nM at rest to 900 ± 606 nM ($N = 11, 5$) in 20 s (Fig. 1*g*), and synaptic potentials were also observed (Fig. 1*h*). Twin pulse stimulation for 90 s in manganese Ringer's produced a 189 ± 55 nM ($N = 8, 4$) increase in $[Ca^{2+}]_i$ (Fig. 1*e*) but, as in ref. 10, no detectable evoked transmitter release (Fig. 1*f*).

Since these calcium-free and manganese Ringer's permit calcium influx during membrane depolarization, this would cause evoked transmitter release which can be facilitated to detectable levels by elevating presynaptic $[Ca^{2+}]_i$, either through high frequency stimulation or the use of photolabile calcium chelators or mitochondrial poisons[10]. Therefore, such release does not support the calcium-voltage hypothesis. We tested this hypothesis by using the photolabile calcium chelator DM-nitrophen and solutions which more effectively prevent calcium entry. We chose DM-nitrophen, instead of nitr-5, because it binds calcium with a higher affinity before photolysis and a lower affinity after light exposure[12], providing bigger changes in $[Ca^{2+}]_i$. We found that a 'cobalt Ringer's' containing 13.5 mM cobalt, 30 mM magnesium, and no added calcium, permits only minimal calcium entry during action potentials. The $[Ca^{2+}]_i$ rose by only 64 ± 19 nM ($N = 11, 5$) during a 20-s 100-Hz stimulation, and no transmitter release was observed (Fig. 1*i*, *j*). The small rise in $[Ca^{2+}]_i$ and the presence of nerve field potentials indicate that action potentials were invading the nerve terminals but that calcium influx was insufficient to evoke transmitter release. A zero-calcium saline containing 2 mM EGTA was even more effective in reducing calcium influx but EGTA often led to presynaptic depolarization and failure of the action potential.

With a solution that effectively prevents calcium influx, it was possible to test the calcium-voltage hypothesis. In normal Ringer's, DM-nitrophen photolysis strongly increased transmitter release, expressed as the frequency of appearance of miniature e.p.s.ps, by 1,700–22,500% ($N = 12$); evoked e.p.s.ps were facilitated by 173–2,188%. These effects are much more powerful than those seen using nitr-5 (ref. 10).

Figure 2*b*–*f* illustrates one of six experiments to test for an effect of voltage on transmitter release without calcium influx. Initially, action potentials elicited large e.p.s.ps in normal Ringer's, in addition to a small nerve terminal potential recorded by the muscle electrode (Fig. 2*c*). After rinsing in cobalt Ringer's for 10 min, 10-Hz stimulation failed to evoke transmitter release, but the muscle electrode still recorded a nerve terminal potential (Fig. 2*d*). Photolysis of nitrophen triggered intense transmitter release, with miniature frequency rising from about 1 Hz to 4,500 Hz (800–10,700 Hz, $N = 6$) (Fig. 2*b*). Nevertheless, action potentials still failed to activate transmitter release (Fig. 2*e*). On return to normal Ringer's the e.p.s.p. reappeared in 3–4 min, smaller in amplitude, but broader than the control synaptic potential (Fig. 2*f*), due to the residual effects of TEA and incomplete exchange of solutions.

These and previous[10] results lead us to the following conclusions about synaptic transmission at the crayfish neuromuscular junction: (1) saline solutions containing magnesium or manganese and low calcium permit calcium influx and presynaptic accumulation during action potentials and produce evoked release at high stimulus frequency; this transmitter release is potentiated by elevation of resting $[Ca^{2+}]_i$; (2) solutions containing cobalt (or manganese or EGTA) and no added calcium allow minimal calcium influx and as a result do not produce transmitter release to action potentials; (3) elevation of $[Ca^{2+}]_i$ in crayfish presynaptic boutons through photolysis of DM-nitrophen causes a large facilitation of evoked transmitter release and increases miniature e.p.s.p. frequency in a normal calcium medium; (4) in solutions which effectively block calcium influx during action potentials, elevation of $[Ca^{2+}]_i$ through photolytic release of 'caged' calcium strongly activates transmitter release, but the depolarization of an action potential produces no additional release; (5) the normal spike-evoked secretion of neurotransmitter is not affected directly by presynaptic voltage but is triggered exclusively by an increase in $[Ca^{2+}]_i$ near transmitter release sites. □

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Nature of the motor element in electrokinetic shape changes of cochlear outer hair cells

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IT is the prevailing notion that cochlear outer hair cells function as mechanical effectors as well as sensory receptors[1–3]. Electrically induced changes in the shape of mammalian outer hair cells[4,5], studied *in vitro*, are commonly assumed to represent an aspect of their effector process that may occur *in vivo*. The nature of the motile process is obscure, even though none of the established cellular motors can be involved[6]. Although it is known that the motile response is under voltage control[7], it is uncertain whether the stimulus is a drop in the voltage along the long axis of the cell or variation in the transmembrane potential. We have now performed experiments with cells partitioned in differing degrees between two chambers. Applied voltage stimulates the cell membrane segments in opposite polarity to an amount dependent on the partitioning. The findings show, in accordance with previous suggestions[6,8], that the driving stimulus is a local transmembrane voltage drop and that the cellular motor consists of many independent elements, distributed along the cell membrane and its associated cortical structures. We further show that the primary action of the motor elements is along the longitudinal dimension of the cell without necessarily involving changes in intracellular hydrostatic pressure. This establishes the outer hair cell motor as unique among mechanisms that control cell shape[9].

Mammalian outer hair cells are slender cylindrical structures of fairly uniform diameter (~8–10 μm) and their length ranges from ~20–30 μm in the high-frequency cochlear base, to about 80–100 μm in the low-frequency apex. These cells are distinguished by the presence of several layers of subsurface cisterns[10,11] and an elaborate network of cortical cytoskeletal elements between the cisterns and the plasma membrane[12–14]. Outer hair cells possess primarily efferent innervation with only a rudimentary afferent endowment[15,16] that constitutes 5–10% of the cochlear outflow[17]. Yet, interference with outer hair cell function produces profound changes in afferent neural and behavioural responses[18,19], implying a mediating effect on